Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2007.03466.x

Antibodies directed against ribosomal P proteins cross-react with phospholipids

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Accepted for publication 15 June 2007
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Summary

Anti-ribosomal P protein (anti-P) antibodies are marker antibodies in systemic lupus erythematosus (SLE). Their association with psychiatric or neurological manifestations has been proposed, but remains controversial. Anti-phospholipid antibodies are the hallmark of a syndrome that may comprise a number of neurological manifestations. Thus, anti-P and anti-phospholipid antibodies have both been associated with central nervous system involvement and their co-existence in the same sera was reported. We verified the ability of purified anti-P antibodies to bind different phospholipids and phospholipid-binding proteins in solid-phase assays. Anti-P antibodies from five of eight patients bound cardiolipin (CL) when saturated with fetal calf serum (FCS); in three cases anti-CL antibodies were also detected in the flow-through. No anti-P eluate, nor any corresponding flow-through, bound β_2-glycoprotein I alone or prothrombin. Moreover, no anti-P eluate bound CL when the plates were blocked with bovine serum albumin in the absence of FCS. Anti-P antibodies with anti-CL activity bound both ssDNA and dsDNA and also nucleosomes in three patients. Our data indicate a great heterogeneity of anti-P antibodies that appear to be overlapped partially with the other autoantibody populations detected frequently in SLE.

Introduction

Anti-ribosomal P protein (anti-P) antibodies are directed to three ribosomal phosphorylated proteins sharing the C-terminal sequence that represents the dominant epitope [1]. Their specificities and clinical associations have been investigated extensively. They appear clearly to be a hallmark of systemic lupus erythematosus (SLE) where they are present in nearly 15–20% of patients, their presence in other connective tissue diseases (CTDs) being occasional. The proposed association with psychiatric or neurological manifestations of SLE is controversial and no definitive conclusion has been drawn so far [2–4].

Anti-phospholipid antibodies are directed against a diverse group of phospholipids and phospholipid-binding proteins; among these, anti-cardiolipin (anti-CL), anti-beta-2-glycoprotein I (β_2-GP-I) and anti-prothrombin antibodies seem to be the most relevant from the clinical viewpoint [5]. Anti-phospholipid antibody syndrome may appear as a stand-alone syndrome or associated with major CTDs (as SLE) and may comprise a number of neurological manifestations [6].

Thus, both anti-P and anti-phospholipid antibodies have been associated with central nervous system (CNS) involvement and their co-existence in the same sera was reported. These sera may contain two separate autoantibody populations or a unique antibody showing a double specificity.

We decided to investigate this point, verifying the ability of purified anti-P antibodies to bind different phospholipids and phospholipid-binding proteins in solid-phase assays.

Methods

Patients and sera

A number of patients fulfilling the criteria for the diagnosis of SLE were screened for the presence of anti-P antibodies in their sera. We selected eight sera containing high amounts of anti-P antibodies [7]. Among them five patients were in an active phase of disease and in four of them anti-dsDNA antibodies were found. Three patients were in remission. Neuropsychiatric manifestations were not present in any of the patients. In six patients IgG anti-cardiolipin antibodies

were detected. The patients gave informed consent to the study.

Purification of anti-P antibodies

Antibodies were affinity purified using a column where the C-terminal amino acid sequence of P proteins was linked [7]. Briefly, the sera were precipitated with ammonium sulphate, each precipitate was dissolved, dialyzed in phosphate-buffered saline (PBS) and then applied to the column. Flow-through was also collected for testing. Different elution conditions were tested: low pH, high salt and dimethylsulphoxide (DMSO) 10%, without any significant difference in the yield and/or specificities of eluted antibodies. Eluted antibodies were then dialysed in PBS. No eluate was obtained from anti-P positive sera applied to a resin coupled to an unrelated peptide.

Enzyme-linked immunosorbent assays (ELISAs) for antibody specificities detection

Anti-P antibodies were detected by ELISA [8]. Briefly, plates were coated with the 13-mer peptide synthesized as multiple antigen peptide at 1 μg/ml, saturated with autoclaved gelatine and incubated with purified anti-P antibodies at 10 μg/ml in gelatine 0·5% Tween 0·05% in PBS. Bound antibodies were detected by goat anti-human IgG (Sigma, St Louis, MO, USA) conjugated with alkaline phosphatase.

Anti-DNA antibodies were detected as described previously [7], on plates precoated with polylysine, coated with 10 μg/ml of ssDNA or dsDNA, post-coated with polyglutamate and blocked with 3% bovine serum albumin (BSA; Sigma) 5% fetal calf serum (FCS; Eurobio, Courtaboeuf Cedex B, France). The antibodies, diluted in 1% BSA 2·5% FCS 0·05% Tween in PBS, were incubated on the plate and after washings alkaline phosphatase-conjugated goat anti-human IgG (Sigma) was added.

Anti-nucleosomal antibodies were detected by a commercial kit (Inova Diagnostic, Quanta Lite Chromatin ELISA, San Diego, CA, USA). Eluates and flow-throughs of the peptide column were tested at 10 μg/ml, according to the manufacturer's instructions.

Anti-CL antibodies were detected by ELISA on polystyrene plates coated with cardiolipin (0·2 mg/ml in ethanol) (Sigma). After blocking with PBS 10% FCS, antibodies diluted in PBS 1% BSA 2·5% FCS were added and the plates were incubated for 4 h at room temperature. After washings, alkaline phosphatase-conjugated goat anti-human IgG (Sigma) was added. Anti-CL antibodies were also detected by a similar ELISA where the blocking agent was BSA, in the absence of FCS.

Beta2-GP-I was purified from normal human sera and anti-β_2-GP-I antibodies were detected by ELISA, as described previously [9], with minor modifications. Beta2-GP-I was used at 10 μg/ml for the coating of Nunc MaxiSorp plates (Nunc, Roskilde, Denmark), BSA 3% in PBS was employed for plate blocking and BSA 3% Tween 0·05% in PBS for antibody dilution.

Anti-prothrombin antibodies were detected according to Matsuda *et al.* [10], with minor modifications. Microtitre plates were coated with 30 μl of 50 μg/ml phosphatidylserine (Sigma), dried overnight and blocked with Tris-buffered saline (TBS) containing 1% fatty acid-free BSA and 5 mM CaCl2 (TBS/BSA/Ca). After washings with TBS Tween 20 0·05% 5 mM CaCl2, human prothrombin (Stago, Asnières sur Seine, France) in TBS/BSA/Ca was added. Antibodies were diluted in the same buffer and incubated on the plate. Alkaline phosphatase-conjugated goat anti-human IgG (Sigma) was then used.

Inhibition tests were performed preincubating anti-P antibodies with various amounts of peptides or proteins (40–2 μg/ml) or ss or dsDNA (100–3 μg/ml) before testing on CL-coated plates.

Positive controls were run within each assay; immunoglobulins from normal human sera were included as negative controls in both binding and inhibition assays.

Results

We tested the purified anti-P antibodies and the corresponding flow-throughs (devoid of anti-P activity) on the different phospholipids and phospholipid-binding proteins. The results are shown in Table 1. Anti-P antibodies from five patients also bound CL in the presence of calf serum (five of eight). In two patients the peptide column removed all the anti-CL activity, while in three cases anti-CL antibodies were also detected in the flow-through. In two patients anti-CL antibodies were present only in the flow-through, while in one patient no reactivity to CL was detected, either in the eluate of the peptide column or in the flow-through. No anti-P eluate, nor any corresponding flow-through, bound β_2-GP-I alone or prothrombin. Moreover, no anti-P eluate bound CL when the plates were blocked with BSA in the absence of FCS, not even when β_2-GP-I was added. In contrast, in one case, the addition of prothrombin restored the binding ability of anti-P antibodies to CL. The binding of anti-P antibodies to CL in the presence of FCS was not inhibited by the P peptide in the liquid phase.

Anti-P antibodies were also tested on DNA (see Table 1): all five anti-P antibodies with anti-CL activity bound both ssDNA and dsDNA and three of them also bound nucleosomes. The binding of anti-P antibodies to CL in the presence of FCS was not inhibited by ss or dsDNA. Among the three anti-P antibodies devoid of anti-CL activity, two were negative on dsDNA while one bound dsDNA and also nucleosomes.

On the whole, of four anti-P antibodies that bound nucleosomes three also bound CL, and among the three flow-throughs containing anti-nucleosome antibodies two also bound CL. The five flow-throughs that contained

Table 1. The table summarizes the results obtained with anti-P and non-anti-P antibodies, testing their binding activity on various targets in solid phase enzyme-linked immunosorbent assay.

	ACLA	ssDNA	dsDNA	Nucleosomes
Patient 1				
Purified anti-P	NEG	POS	POS	POS
Flow-through	POS	POS	NEG	NEG
Patient 2				
Purified anti-P	POS	POS	POS	POS
Flow-through	POS	POS	NEG	NEG
Patient 3				
Purified anti-P	POS	POS	POS	NEG
Flow-through	POS	POS	NEG	NEG
Patient 4				
Purified anti-P	POS	POS	POS	POS
Flow-through	POS	POS	NEG	POS
Patient 5				
Purified anti-P	POS	POS	POS	NEG
Flow-through	NEG	POS	NEG	NEG
Patient 6				
Purified anti-P	NEG	POS	NEG	NEG
Flow-through	NEG	NEG	NEG	NEG
Patient 7				
Purified anti-P	POS	POS	POS	POS
Flow-through	NEG	POS	NEG	POS
Patient 8				
Purified anti-P	NEG	POS	NEG	NEG
Flow-through	POS	POS	NEG	POS

Anti-cardiolipin antibodies (ACLA) were of IgG class. NEG: no binding activity, POS: presence of binding activity. All the eluates and the flow-throughs were also tested on β2-GPI and on prothrombin: no binding activity was observed.

anti-CL antibodies were all devoid of anti-dsDNA activity, but all bound ssDNA antibodies and two of them also bound nucleosomes.

Discussion

Anti-P antibodies are a hallmark of systemic lupus and in the past they have been related to CNS involvement of lupus patients. Subsequent studies, however, did not confirm these observations and this relationship is still a matter of debate [2,3,11,12]. Anti-P antibodies were also related to renal involvement in SLE patients such as anti-dsDNA [13,14]. This association with active nephritis may be due, at least in part, to the overlap of anti-P and anti-dsDNA antibodies. In previous studies, in fact, we showed that anti-P antibodies cross-react with other SLE autoantigens such as Sm and DNA [7,15]. Subsequently, a relationship of anti-P with autoantibodies not restricted to SLE, such as anti-CL, was suggested [16,17]. We investigated this relationship using affinity purified anti-P antibodies tested by ELISA on different phospholipid targets, on DNA and nucleosomes.

We found that some anti-P reacted with CL in the presence of FCS (containing β_2-GP-I), while no binding activity was found towards the proteins β_2-GP-I or prothrombin or to CL alone. The addition of prothrombin in the absence of FCS restored the binding ability to CL, while the addition of β_2-GP-I was not effective, thus suggesting that other serum proteins may complex with CL and become a target for anti-P antibodies.

Some anti-phospholipid activity was not extracted by the peptide colum and remained in the flow-through.

All five anti-P antibodies that bound CL-β_2-GP-I were also able to bind both ssDNA and dsDNA, while the five flow-throughs reactive with CL-β_2-GP-I were all devoid of anti-dsDNA antibodies, but all contained anti-ssDNA antibodies.

The ability of anti-P antibodies to bind dsDNA, nucleosomes and CL-β_2-GP-I may be due to a single antibody molecule displaying multiple binding abilities or to different antibodies, each reacting with a single antigen but all sharing anti-P specificity. In either case, it is remarkable that antibodies purified on a short amino acid sequence are able to bind so many lupus autoantigens. The binding of anti-P antibodies to CL in the presence of FCS, as observed previously for binding to DNA [7], was not inhibited by the P peptide in the liquid phase. These results suggest that the antibody binding site can accommodate both antigens, as the binding of one antigen does not restrict the binding ability for the other antigen.

Among the three anti-P antibodies not able to bind CL, two did not bind dsDNA while one did: this latter also showed anti-nucleosome ability. In two flow-throughs containing anti-CL antibodies, anti-nucleosome antibodies that did not react with dsDNA were detected. These results suggest a different fine specificity of anti-nucleosome antibodies contained in the anti-P eluates or in the flow-through.

Associations between anti-P and anti-CL antibodies have been reported recently [14,16,18], and we also observed the co-existence of anti-P and anti-CL in the same lupus sera [17]. At variance with our results, previous attempts to show that a cross-reactivity of anti-P purified antibody with CL had failed [16]. The different results may be due to the different strategies used to purify antibodies: Ghirardello *et al.* [16] eluted anti-P antibodies from nitrocellulose-bound antigen while we immobilized the immunodominant peptide on a chromatography column. This procedure may be more efficient in the extraction of anti-P antibodies, thus allowing detection of more binding specificities.

In conclusion, the data presented in this paper, together with our previous findings, indicate a great heterogeneity of anti-P antibodies that are overlapped partially with the autoantibody populations detected more frequently in SLE. These cross-reactivities may contribute to understanding of the possible pathogenic role of anti-P antibodies.

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